FILTERABLE FORMS OF BACTERIA

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Numerous authors have reported the occurrence of filterable elements in cultures of bacteria. These elements which were, of course, viable, passed through filters which held back the normal forms. Most of the records on the filterable forms were based on filtration experiments with emulsified cultures or pathogenic material. The filtrates were tested either in laboratory media or by injection into animals, and the results were called positive, if a culture grew or a reaction in the animal was observed. The reports were at first received with the widest interest, but on repetition many workers were unable to obtain positive results. Those which had first proclaimed the existence of a filterable phase could not answer the challenge of the newcomers by devising well defined conditions under which positive results occurred with reasonable frequency. Therefore the interest in the problem gradually subsided. It is significant that newer bacteriological books such as Dubos' book on the bacterial cell (38) do not mention the word filterability in connection with bacteria.

The object of this article is to give an account of recent studies dealing with the L phase of bacteria and to follow this up by a review of the work done on the filterable forms of bacteria. It must be stressed that the L phase was first discovered in *Streptobacillus moniliformis* and is here regarded as a phase of bacteria which produces a large number of small filterable viable elements. It is felt that the facts which have been revealed in regard to the L phase shed a new light on the problem of filterable forms in bacteria and give new significance to a wealth of valuable information already reported in the literature.

THE FILTERABLE L PHASE IN BACTERIA

(*a*) *History. Streptobacillus moniliformis* is a slender, gram-negative, pleomorphic bacillus which grows in chains. It causes rat-bite fever in man (40, 11) and was isolated from cases of "Haverhill fever" (Parker and Hudson, 119). It is responsible for an infectious arthritis in mice (94, 100) and leads a more or less saprophytic existence in the nasopharynx of rats (127; review by Sabin, 123). In 1935 it was demonstrated for the first time by me (82) that a growth which did not contain bacillary forms, but was composed of soft cytoplasmic elements, globules and minute granules could be separated from an *S. moniliformis* culture. This new type of growth was designated by the letter L, and that of *S. moniliformis* in particular by L 1. The new growth phase was isolated by me (83) from all *S. moniliformis* strains tested, and later from *Spherophorus necrophorus* (86) as well. The L phase strains bred true after a number of purifications. I realized that two interpretations were possible, viz., (a) that the L form is a growth phase of *S. moniliformis*, or (b) that the L form is a symbiont genetically different from the bacillus. The first interpretation seemed very audacious at the time and full of far reaching implications; for this reason and also because of

the striking similarity of the L growth in morphology, growth requirements and colony type with the organisms of contagious pleuropneumonia of cattle and agalactia of sheep the second interpretation was adopted. All the later workers in the field (11, 13, 16, 17, 18, 27, 69, 116, 125) challenged this interpretation and adopted the first one. In favor of my view was the fact that from the mother culture, L strains could be obtained which bred true and could not be induced to revert into the bacillary form; in favor of the other side was the fact that the mother culture always seemed to be mixed with the L growth which could not be eliminated. There were also strains of the L type that were very difficult to purify and which reverted again and again. As an example I have isolated two new L strains recently, one from a fresh *S. moniliformis* culture, the other from *Spherophorus necrophorus "132"*, both of which reverted to the bacillary form, the first one until the 35th subculture, the second one until the 40th.

Convincing evidence in favor of the growth phase interpretation was brought forward by Dienes (22) and Dienes and Smith (32, 33) who showed by means of warm stage observations that the big L elements divided up and finally reproduced bacilli. I confirmed this (87) and in the same paper produced evidence to indicate that whenever the L form arises this process is preceded by a fusion of small elements. Therefore I am now in agreement with the other authors who regard the L form as a phase of the bacillus. In a later paper, I (89) brought forward additional evidence by use of the phase microscope to show that bacteria produce small granular elements which seem to fuse. This fusion is followed by the rapid development of the L bodies. I am therefore of the opinion that the L cycle may be regarded as a process of regeneration in bacteria probably equivalent to a sexual process in higher organisms. It is very likely a general feature in bacteria. It is particularly to Dienes' credit to have demonstrated the L phase in a large variety of different species such as *Flavobacterium* and *Haemophilus influenzae* (21, 27), *Bacterium funduliformis* (17, 18, 20, 28), *Escherichia coli* (19), *Haemophilus para-influenzae* (23), *Proteus vulgaris* (24, 25, 34) *Salmonella typhi* (29, 35), *Salmonella* and *Shigella* (136), organisms of the genus *Bacillus* (30) and *Clostridia* (31).

(*b*) *Nature and significance.* The L phase is not comparable to other bacterial dissociative variants such as the R-form or the S-form, for it is an entity of its own as different from bacteria as the tadpole from the frog. In a gram-stained smear of an L culture only a faint cloudiness can be discovered. Its cytoplasmic elements are amorphous in that they can take any shape and can be of large or very small size. Löhnis' name "symplasm" and Almquist's "plasmodium" are very appropriate designations for these forms. They can transform directly into bacilli or can (and always do in the "stabilized" strains which breed true) reproduce their own kind by segmentation and by the production of minute granules which can grow out again into the cytoplasmic elements. Thus the developmental cycle is essentially simple in a stabilized L culture. A similar development takes place in the organisms of the pleuropneumonia group as shown by Klieneberger and Smiles (90). The morphology of the L growth has been widely studied. Serological studies have been carried out by me (85) and by Dienes and collabo-

rators (34). In regard to pathogenicity very little work has been done so far; I was not able to infect mice with the L form of a pathogenic *S. moniliformis*. However a stabilized L culture was once isolated from the lung of a rat suffering from bronchopneumonia (84). It has been found that the L phase is resistant to penicillin, though the homologous bacterial phase is sensitive (27 to 29).

The first observable change in cultures about to produce L forms is that some bacteria become transparent and less stainable. Then they either transform into a chain of small granules or form granules at their tips and in other areas of their body. These granules can remain in connection with the bacterium or they can become free (89). Two (or more) of these granules seem to unite and grow rapidly into L bodies. At or before the stage of granule formation many bacteria show characteristic arrangements of their cells. For example in *Bacterium Morax-Axenfeld* "260" (89) and in *S. moniliformis* (88) twisted filaments are frequently developed. In many organisms loops, skeins or ball-like entanglements are formed. When the small granules, which have combined, remain connected with the mother bacterium, then the impression created by the growing L body is, that the bacteria themselves swell up. Yet if the granules are free, they can often be seen in couples, and when joining they form at first small triangular, square or oblong bodies. These as well as the bodies derived from loops and twisted filaments suggest the occurrence of fusion.

The particle size of the granules of the L phase of *S. moniliformis* has been analysed (88). A fresh culture of *S. moniliformis* and an L culture carried in the stabilized stage for 14 years called "L 1 rat 30" were used.

Two day old serum broth cultures of the L strain were centrifuged, the sediment ground thoroughly and resuspended in serum broth. This emulsion was centrifuged a second time in an angle centrifuge at 4000 rpm for 10 minutes. The slightly opalescent supernatant contained fine granules which could be demonstrated (though with difficulty) by staining. This suspension was filtered through the following gradocol membranes: Average Pore Diameter = 700, 600, 500, 400, 350 mμ. The positive pressure was approximately 10 cm. Hg and the time of the filtration 5 to 10 minutes. Both the opalescent suspensions and the clear filtrates were titrated by placing measured and diluted amounts in tubes of serum broth which were incubated. The filtration experiments with the *S. moniliformis* culture were carried out with 18 hr-old cultures, which were sedimented, ground, resuspended and finally centrifuged for 2 to 3 minutes at 2000 rpm. Here membranes of 900 and 800 mμ were used as well as the finer ones.

In the case of the L strain it was found that the supernatant contained between 1 and 10 million particles per ml which grew out into L colonies; the filtrates which had passed the 700, 600, 500 mμ A.P.D. membranes contained 10,000 to 100,000 viable particles per ml. The 400 mμ filter also allowed elements to pass, yet here a drop in particle number had occurred; about 10 particles per ml had passed the membrane. No particles passed the 350 mμ filter. Therefore the end point of filtration had been reached and, according to Elford's formula (88) the particle size would appear to range from 175 to 250 mμ. Consequently, the small reproductive granules of the L phase of *S. moniliformis* are slightly

smaller than the particles of psittacosis virus and slightly larger than the elementary bodies of vaccinia virus and the minimal reproductive units of the organism of bovine pleuropneumonia. The elements of the Streptobacillus passed the 900 and 800 mμ membranes but not the 700 mμ filter. Therefore the average size of the bacillary forms is 750 mμ, the size usually attributed to *Serratia marcescens*. It must be emphasized that such an assay can only be carried out with a stabilized L strain that has been well adapted to grow on artificial media. During a prolonged initial period newly isolated L cultures can only be propagated by mass inoculations. It may take many years of passages to produce a strain which will grow from a single granule inoculum. Two new strains, one of *S. moniliformis* and the other of *Spherophorus necrophorus* origin, were isolated two years ago and are still not well enough adapted to grow from a single granule inoculum, however, they grow gradually from smaller and smaller inocula. The small granules have been observed by most investigators; their importance, however, has not been generally recognized, except by Tulasne (130, 131, 132) who drew attention to these "formes submicroscopiques". It should be pointed out that they are not truly submicroscopic, because if properly stained, they are demonstrable with the light microscope. They can be cultivated on special very rich media, and they are filterable. The filtration analysis has established their viability beyond doubt, so that they can no longer be interpreted as nonviable products of disintegration or autolysis as Dienes (25) and Freundt (46) have suggested. Furthermore, the regular production of viable granules by the large and small bodies rules out the validity of Ørskov's (117) interpretation, that they are abnormal forms produced by conditions extremely unfavorable to growth ("involution forms"). Slightly adverse conditions are frequently (not always) the causative agents of production of L forms. By means of the fusion, which may bring about regeneration, new bacteria may arise, which may be better adapted to the adverse factor. Though the L cycle seems established, our knowledge of the phenomenon is still fragmentary, and many puzzling questions remain to be answered. For example: Would the stabilized strains revert if the right conditions were applied? Is the L phase of bacteria pathogenic? When the L forms of *S. moniliformis* and *Spherophorus necrophorus* were first isolated in pure culture, it was believed that their occurrence in ordinary bacterial cultures presented a hitherto unknown fact. Yet with closer study of the phenomenon it gradually becomes clear that innumerable authors through decades of bacteriological literature had been describing "aberrant" bacterial morphology, "big bodies" and granules which were aspects of the L phase. Since their interpretations were manifold, the subject has become one of the most controversial in bacteriology. It is believed that the facts which have emerged from the studies of the L cultures (scanty as they may still be) shed a new light on the vexing problems of bacterial pleomorphism and granular filterable forms. We therefore propose to review the pertinent literature in order to determine whether a uniform interpretation of these elements as part and parcel of the L cycle may not bring in line many of the scattered factual observations.

FILTERABLE FORMS IN SPIROCHETES

Nicolle and Blanc (113) and Nicolle (111) during their work on relapsing fever conceived the idea that spirochetes must exist in a visible and an invisible stage. They observed that after a louse had fed upon a patient, infected with spirochetes, the parasites traversed the cells of the intestine of the louse in the first few hours, but then the parasites remained undemonstrable until the sixth or seventh day when they reappeared in the insect, but were extremely small. They gradually increased in size until finally they reached the size of the adult spirochetes. The virulence of the louse spirochetes was at its highest on the sixth day after the blood meal when the actual parasites were either still invisible or very minute. After the eighth or ninth day the louse-spirochetes lost their virulence completely. According to Nicolle (112) and to Nicolle and Anderson (114) the relapsing fever spirochetes occur in the louse in two alternating forms, one avirulent and visible, the other virulent and invisible.

Relapsing fever in the patient runs a course characterized by a repetition of fever attacks which become less severe until recovery takes place. The resolution of the crisis is sudden. The spirochetes which have become very numerous disappear from the blood in a few hours. Nicolle's interpretation of the characteristic evolution of the disease is that the parasites go into a granular stage produced by fragmentation of the adult forms. The granular stage is resistant and persists in the tissues. The repetition of the fever is brought about by an invasion of the blood by "previsibles" spirochetes which are fully virulent and which develop into the large adult form.

Hauduroy (65) reviews Leishman's investigations of a tick-borne infection of monkeys. The spirochetes went through a cycle in the ticks. The day after the intake of infected blood they were found agglutinated inside the digestive tube of the tick. Gradually they underwent fragmentation, and granules of different sizes were liberated into the intestinal tract. The granules became dispersed in the tick. Leishman observed heaps of granules as well as small, very young spirochetal forms in the ovarium of a tick. He found that emulsions of ticks in which spirochetes had not been found by microscopical examination, caused infection in the monkeys. Prowazek, Blanc, Brumpt, Wolbach, Marcloux (65) confirmed Leishman's observations, and all these authors stated that the spirochetal cycle includes an "invisible stage". According to Hauduroy, *Borrelia recurrentis*, *B. duttoni* (African tick fever) and *B. venezuelensis* (American tick fever) have been shown to pass china filters which retain ordinary bacteria.

It is most interesting that in the cycle of spirochetal evolution a phase seems to occur in which the organism persists in the form of small granules. This form is apparently resistant and latent and becomes infective when it regenerates spirochetes. Yet the orthodox school interprets the disease picture solely on the basis of serological observations. According to the textbooks the relapsing fever spirochetes disappear from the blood by the destructive action of immune bodies. It has been found that in the organs of the body the antibody titre is less high than in the blood; therefore it is believed that some spirochetes are

able to remain latent in the brain and other tissues. There they undergo an antigenic change and become resistant to the host antibodies. They invade the blood again in the form of a serum-fast strain and stimulate the host to the production of antibodies directed against the new antigenic variety. These new antibodies are the cause of the second disappearance of the spirochetes from the blood. Yet a third antigenic change and a third relapse may occur (6).

It might be thought that the two explanations are contradictory, but this is not the case. Rather they complement each other and are in perfect harmony. It is known that growth in immune serum causes organisms to go into the L phase. Through the process of regeneration they may emerge as organisms resistant to the inhibitory serum factor. It is therefore feasible that in spirochetes an antigenic as well as a morphological transformation occur at the same time.

An evolutionary cycle for *Treponema pallidum* has been suggested by Levaditi, Schoen and Sanchis-Bayarri (93) who studied the morphology of the organism quite extensively. They observed that in lymphatic glands of rabbits, infected by the scrotal route, spirochetes were very rarely found microscopically, although the glands were infective for new animals. By microscopical examination of these glands it was found that the spirochetes which invaded the glandular tissue were gradually replaced by shorter and denser spirochetes. It is difficult to form a clear picture of the sequence of events from the author's description:

> "La forme nettement et régulièrement spiralée est remplacée progressivement par des spirochètes plus courts, plus épais et moniliformes; ceux-ci se raccourcissent davantage et montrent des renflements à l'une ou aux deux de leurs extrémités. Par la suite, le parasite se dispose en boucles, d'abord incomplètement fermées, ultérieurement plus complètes. Plus tard, et après s'être disposé en pelotes serrées, le tréponème prend l'aspect d'une virgule, puis se transforme en granulations rondes, ovolaires ou irrégulières. Celles-ci finissent par devenir si petities qu'elles ne peuvent être distinguées que très difficilement."

According to Levaditi the granular form represents the pre-spirochetal phase of the syphilitic agent. The granules are able to retransform themselves into young spirochetes and then into the long, spiral adult form. The granular form persists in the tissues during periods of latency and withstands specific treatment. Although isolation of the granules by filtration would give final proof of the existence of the granular stage in the tissues, Levaditi and collaborators were not able to obtain positive filtrates through filter candles.

Levaditi's conception would be in agreement with the fact that spirochetes are not found in certain diseased tissues, that they are not demonstrated in nerve fibres from cases of paralysis of the insane and of tabes and that latent stages of the disease resist chemotherapeutic treatment.

Simon and Mollinedo (124) investigated the cycle of *T. pallidum* by serial punctures of the lymphatic glands in cases of syphilis during the disease and treatment. They found that *T. pallidum* underwent a transformation and that one of the stages of the cycle was granular, ("granule spirochétogène"). This granular stage persisted in the glands for a long time during chemotherapeutic treatment. According to the drug applied the adult spirochetes decreased more

or less rapidly. The authors believe, that a cure is not achieved unless the granular stage disappears from the human organism as well.

A promising new approach to reveal the life cycle of *Borrelia duttoni* has been made by Levaditi, Vaisman and Chaigneau (95). The authors infected developing chick embryos with spirochetes from the blood of a mouse. The infection killed the embryos before the eighth day, at a time when spirochetes had developed abundantly. The eggs were opened on the 5th, 6th, 7th, 8th and 15th day after inoculation. Smears from the allantoic membrane and the organs were studied under the ultramicroscope and in silver impregnated and Giemsa stained preparations. Four successful passages in eggs have so far been carried out. The morphological studies revealed the presence of a remarkable amount of "atypical" forms, manifesting themselves particularly in liver and spleen. Balls, loops and argentophilic almost submicroscopic granules were observed in abundance. They occurred in the interstices between the cells as well as inside the cytoplasm of the cells themselves. The authors expressed the opinion that the argentophilic granules are able to regenerate the typical wavy spirochetes. They believe that the cycle observed is of general significance and resembles the cycle described in 1928 by Levaditi and his collaborators for *T. pallidum*. It can be regarded as an important progress that the cycle has been observed in the developing egg. Future continuous studies in tissue culture should be carried out and watched under the phase microscope.

In recent studies DeLamater, Wiggall and Haanes (14, 15) reported their observations on the life cycle of *T. pallidum* in the living rabbit testis. Their evidence is obtained from phase contrast observations and stained preparations. They saw actively motile spirochetes "come together in pairs and complex clusters." It seems that after a fusion of some parts of these spirochetes, cysts arise in which "dense basilar granules" are seen. Apparently, these can give rise again to young spirochetes which emerge from the cysts. DeLamater expresses the view that a particular stage of the developmental cycle of the spirochetes might be responsible for the latency phenomenon in syphilis.

Hampp, Scott and Wyckoff (60) investigated cultured strains of oral spirochetes with the electron microscope. They demonstrated free granules and big bodies; the fate of the latter has not been further investigated. From the illustrations no information is obtained as to whether they may have been produced by a process of fusion.

The details of the life cycles of the various spirochetes have not as yet been completely revealed. However, the evidence shows that their developmental cycle may well be similar in essential features. There seems to exist an alternation between the actual spirochetal phase and a granular phase which, it is assumed, may represent the regenerative or L phase. This latter phase is at the same time resistant and responsible for the periods of latency. It is able to reproduce young spirochetes which may in various ways differ from the preceding generation. The existing information indicates that the spirochetal L phase consists of particles which are almost submicroscopic.

The reader is referred to an article by Mollinedo (108) on the developmental cycle of spirochetes containing an extensive list of pertinent literature.

FILTERABLE FORMS IN ACID-FAST BACTERIA

When filterable forms of bacteria were first described, great interest was aroused by the reports which claimed their existence in acid-fast bacteria and in particular in tubercle bacilli. It seemed as if some of the mysteries in regard to the clinical course of the disease might be near solution. A number of excellent reviews have been written on the subject such as Hauduroy's (65, 66) and Topley and Wilson's (129) to which the reader is referred for much of the literature. The problem of a granular phase in acid-fast bacteria will be discussed in two sections: (a) filtration experiments, (b) morphological studies.

(*a*) *Filtration experiments.* Many authors (8, 36, 37, 67, 109, 133, 134) followed the lead of the pioneer, Fontès (45), and filtered emulsions of pathogenic tuberculous material or bacilli through Berkefeld or Chamberland candles. The filtrates were tested either in animals or in laboratory media. If guinea pigs were used, they often showed some reaction, such as loss in weight, rise of temperature or enlargement of glands, but the condition was non-specific for tuberculosis. In those rare cases, in which a generalized typical tuberculosis developed, a flaw in the experiment can usually be found. For example, Durand and Vaudremer (37) took material from a cold tuberculous abscess, ground, emulsified and filtered it and injected the filtrate into two guinea pigs. One developed an orchitis which broke. The pus was injected into another guinea pig which succumbed with classical tuberculosis. Isolated positive results such as the above are open to objections. Morim and Valtis (109) worked with the acid-fast bacillus of Johne, infectious for rats. They injected filtrates into these rodents which died without symptoms. Acid-fast organisms were found in their organs. This apparently positive result does not carry much weight, for Cooper and Petroff (12) claimed that they found acid-fast organisms in the glands of five out of fifteen normal guinea pigs.

Hauduroy and Vaudremer (67), Vaudremer (134) as well as Bezançon and Philibert (8) transferred their filtrates into various media and obtained growth; however, their cultures were atypical and consisted of mycelial elements studded with granules. Mellon and Fisher (106) cultured a soft, moist, mucoid, pigment producing, non-acid-fast organism from the filtrate of an acid-fast saprophyte which produced this non-acid-fast variety by dissociation again years later. Positive results such as these can be confronted with a great number of negative ones (e.g., 3, 12, 53, 120, 121, 135, 126). Most authors used filter candles but the last mentioned ones used gradocol membranes.

In brief: When filtrates were injected into animals and positive reactions were obtained, they were non-specific and inconsistent. When filtrates were transferred to media and cultures were obtained, they were rarely typical tubercle bacilli; moreover, the cultures that were obtained by the different investigators varied from non-acid-fast granular and filamentous cultures to non-acid-fast pigment producers and other saprophytes. On the other hand, the negative results by

no means disprove the existence of a filterable phase. For the filterable stage of the tubercle bacillus might in its relationship to the ordinary tubercle bacillus behave like an L culture of *S. moniliformis* to its bacillary parent form. In the latter, non-adapted L strains cannot as yet be grown from the filtrate, whereas adapted strains grow well after filtration. Furthermore, the L strain is characteristically distinct from the bacillary phase of *S. moniliformis*. It may well be that the filterable phase of the tubercle bacillus is, like the L form of *S. moniliformis*, very difficult to grow from a filtrate. However, if it does grow, one would expect it to be a tubercle bacillus L phase different from the bacillary phase, yet of constant characteristic appearance and properties. Only if cultures obtained from filtrates—aberrant though they may be—are of one and the same appearance and have similar characteristics can filtration experiments be accepted as proof for the existence of a filterable stage.

(*b*) *Morphological studies.* Much (110) made the interesting observation that acid-fast bacteria are rarely demonstrated in the lung nodules of cattle suffering from Perlsucht. The same applies to cold abscesses of human beings. Yet very small amounts of these nodules produced tuberculosis in laboratory animals. If minute amounts of the tuberculous nodules were inoculated on media, cultures of tubercle bacilli were easily obtained. By particular staining methods Much was able to show that two kinds of "tuberkulöses Virus" existed in the lesions: (a) bacilli which were sometimes granulated, and (b) a granular form composed of very small elements arranged singly and in clusters. The granular form was virulent and sometimes was the only form occurring in tuberculous lesions. The material from the nodes in cases of Perlsucht of cattle showed either granules exclusively, or numerous granules as well as fine rods, when stained according to Much; yet it did not contain acid-fast elements. When cultured the granules transformed into fine rods, the fine rods into bigger bacilli and on the sixth day acid-fast bacilli were found in the culture. Much obtained corresponding results in sections of tuberculous material from sheep, pigs and men. The granules and fine rods were gram-positive but not acid-fast; there were transition forms between the granules, the fine rods and the typical acid-fast bacilli. Much also seeded tubercle bacilli into sterile milk from an immunized cow and observed, how gradually a transformation into granules occurred. Later, after the addition of glycerin to the milk-medium, acid-fast bacilli reappeared.

There are many reports in the literature about non-acid-fast organisms and granules in "normally" acid-fast cultures. According to Hauduroy (66) the pellicles formed in liquid cultures of tubercle bacilli always contain both acid-fast and non-acid-fast bacilli. The first are stained red, the second blue by Ziehl-Neelsen's staining method. The blue form is supposed to represent the young stage, the red form the adult stage. In peptone water inoculated with a fragment of a culture from potato medium, Hauduroy (65) saw a fine granular deposit develop while the supernatant remained clear. If shaken up the deposit was seen to consist of a kind of mycelial growth. This mycelium and the numerous granules which the deposit contained in addition were not acid-fast. Hauduroy was able to propagate this granular culture. Grasset (54) used tuberculous material which

had been frozen several times at low temperature. After centrifugation the supernatant was called "Lysat centrifugé"; it contained only non-acid-fast material which consisted mainly of granules. Cultures were obtained from it and by transferring the growth every second or third day, it was carried on to the 120th passage. Though there were still many granules and non-acid-fast elements present in these cultures, the number of acid-fast bacilli increased gradually through these transfers. Karwacki (77, 78, 79) observed acid-fast and non-acid-fast and coccal forms in cultures of tubercle bacilli. Pleomorphism has been observed by many workers. Recently Fitzgerald and Bernheim (43) studied the influence of salicylic acid on the morphology of the tubercle bacillus; they observed aberrant forms but relatively few granules. Pagel (118) found during histological studies many granules in old caseous and calcified lesions and in foci with recent exacerbation.

Granular and non-acid-fast stages in cultures of acid-fast bacteria have also been described (107). Lindegren and Mellon (96) postulated the existence of a particular not very convincing sexual cycle in *Mycobacterium tuberculosis* in order to give a "hypothetical explanation" for the aberrant forms observed. Mellon and Fisher (106) have also worked with an acid-fast saprophyte which they described as "teeming with granules" and which varied in acid-fastness. Alexander-Jackson (1, 2) used her specially devised triple stain technique to show the acid-fast and non-acid-fast organisms in original material from lesions in tuberculosis and leprosy. She found in six neural and tuberculoid cases of human leprosy, presence of zoogloeal forms and absence of typical rods. She observed in some cases granules and acid-fast rods lying within "a blue-staining zoogloeal mat". In preparations from skin biopsies in leprosy she also demonstrated non-acid-fast rods, granules and zoogloeal masses. Hauduroy and Tanner (68) showed by an improved and modified Alexander-Jackson technique a "cycle tinctorial" in the tubercle bacillus. Though a clear picture of the developmental cycle of the tubercle bacillus and related organisms cannot be obtained from the descriptions referred to, it seems possible that elements of an L cycle of acid-fasts have been observed by the various authors.

One of the most fascinating contributions to the problem of the small filterable forms in acid-fast bacteria since Much's discoveries has been made by Morton Kahn (73, 74). He watched the development of small groups and of single micromanipulated tubercle bacilli in microdroplets of Long's medium. The main points of his observations are these: After a few hours of incubation a single, previously homogeneous rod showed certain zones of darker appearance, centrally, subterminally or at both poles. Twenty four to 72 hours after the beginning of incubation cleavages occurred. Three or four separate ovoid units were formed by segmentation; these ovoid units divided further into diplococcal forms. By still further subdivision the ovoid units divided into tiny cocci or granular forms which then clumped together in tight masses. Eventually delicate rods sprouted from the periphery of these clumps. The granules and the first delicate rods were not acid-fast. Gradually these rods increased in length and width and thus produced the mature bacilli which were acid-fast.

This evolutional cycle described by Kahn for the tubercle bacillus has features in common with the L cycle. From the bacteria small granules arise and by clumping may well go through a fusion stage out of which new bacilli are produced. Kahn's publications started a controversy. Gardner (51) observed only a normal multiplication on agar blocks. Ørskov (115) regarded Kahn's small forms as produced through processes of disintegration and crystallisation. Yegian and Porter (138) pointed out how artefacts can be produced through the mechanical action of the platinum loop and the microtome knife. Wyckoff and Smithburn (137) observed in *M. phlei* the gradual appearance of smaller and smaller organisms, but found no evidence of a developmental cycle such as the one described by Kahn. Kahn (75) and Kahn and Nonides (76) repudiated these challenges in two further papers, and in a study of sections through colonies of *M. tuberculosis* presented additional evidence to support their view that the granules and non-acid-fast rods are the youngest forms of the cycle. The studies of the opposing authors have, so it seems, produced evidence completely beside the point, for certain organisms and strains go easily through their L cycle others with difficulty and both only under certain conditions; therefore as long as studies are not carried out with the same strain under the very same conditions as those of Kahn's no conclusions can be drawn from them as to whether Kahn was right or wrong. Kahn's continuous observations have, in my opinion, been carried out with great caution and his illustrations are clear and convincing and do not give the impression of being "artefacts".

Quite recently a new attack on the problem of a life cycle in tubercle bacilli has been launched by Brieger (10). His approach to the problem is a new and promising one. The dissimilarity between *in vivo* and *in vitro* infection experiments in rabbits led Brieger to conclude that in the lesions and in the tissue culture two different stages of the agent of tuberculosis may be present.

In order to show how the "invisible" granular stage reproduced bacteria, Brieger did some direct observations on droplets of amniotic fluid from chicken embryos. He had observed that when tubercle bacilli are injected into the amniotic fluid of a nine day old chick embryo an exceedingly small number of tubercle bacilli were found after three further days of incubation. If the membranes and amniotic fluid were taken out at that stage and incubated in Kirchner's medium "a seething mass of bacilli" develop in a few days time. Brieger chose for his observations the amniotic fluid from a nine day old chicken embryo which had been infected with tubercle bacilli and had been subsequently incubated for another three days. He watched the reappearance of the bacilli in micro-cultures, consisting of droplets of this amniotic fluid. He used for his purpose all available modern methods. At first acid-fast bacilli could hardly be found in the amniotic fluid, but "a peculiar kind of foci" was seen and "a non-organized substance which, however, showed some kind of structure". He continues: "After a few days incubation *in vitro*, we found the same structures present, but they now contained a number of very small elements which were more like dots or tiny microrods, coccoid elements, often described as granules. Soon the whole focus was one mass of these tiny elements which were at first

non-acid-fast, but gram-positive and staining with the modified Giemsa method a distinct blue. On the third day of incubation the tiny pale bluish micro-rods were very predominant. Long branching filaments had grown out of the granular foci." It seems that the developmental cycle cannot yet be clearly understood, but the studies are being continued by Brieger and more facts may come to light revealing more of the cycle of the tubercle bacillus and its granular phase.

Though the information is still incomplete it seems likely that the tubercle bacillus can go through a cycle which includes a granular phase. This brings it in line with the organisms which go through an L cycle. We may well suspect that here too a process of regeneration, manifesting itself by the fusion of small cellular elements, does occur. Furthermore, it is hoped that it will be learned how to grow the granular phase of the tubercle bacillus. Then it should be possible to analyse its filterability in a convincing way that is no longer liable to the criticism and objections justifiably raised so far.

FILTERABLE FORMS IN VARIOUS BACTERIA

The problem of a granular filterable phase in bacteria is connected with the problem of life cycles and variability. The observations made have been interpreted variably; the object of this chapter will be to consider whether the findings can be explained in a similar way as those described for the spirochetes and acid-fast bacteria. For much of the literature the reader is referred to the following reviews and books: Löhnis (97), Enderlein (39), Hauduroy (65), Henrici (70), Hadley (55, 57, 58), Klieneberger (81), Mellon (105) and Braun (9).

Here again it will be convenient to discuss separately experiments based mainly on filtration, and experiments based mainly on morphological studies.

(*a*) *Experiments mainly based on filtration.* Friedberger and Meissner (48) inoculated guinea-pigs, animals known to be resistant to generalized typhoid infection, with organs from a patient who died from typhoid fever. The guinea pigs developed a temperature but no lesions. Typhoid bacilli could not be recovered from them. The temperature producing agent could be transferred in succession to a series of guinea pigs. With the organs of these guinea pigs typhoid antibodies were produced in rabbits. The pyrogenic and antigenic agent was shown to be filterable through Berkefeld filters by Friedberger (47). The authors conceived the idea that an invisible and uncultivable stage of the typhoid bacillus had developed in the guinea pigs. They called it kryptoantigenic virus in contrast to the bacilli, the phaneroantigenic form. Gildemeister and Herzberg (52) were not able to confirm Friedberger's results.

Fejgin (41, 42) carried out similar experiments. She lysed typhoid bacilli by bacteriophage, filtered and injected the filtrates into guinea pigs. A slight reaction (rise of temperature and hypertrophy of organs) occurred in the animals. Her results, though in agreement with Friedberger's, do not seem conclusive.

Hauduroy reported two interesting investigations (63, 64). Previously he had described with d'Herelle (71) that under the influence of bacteriophage the typhoid bacillus could break up into small filterable granules. He undertook a search for resistant, granular bacterial forms "dans la nature". He used samples

of spring water which by routine bacteriological tests had been proved to be polluted. These waters were filtered through Chamberland L 3 candles. One portion of the filtrate was left as it was; the other was transferred to peptone water. Both portions were incubated at 37 C for about eight days, when at the bottom of the tubes little whitish granulations were observed. The microscopic examination showed amorphous matter and granules of various sizes. These cultures grew very poorly; even after months of incubation their aspects had hardly changed. Transfers were only obtained with great difficulty. Albuminous media were of advantage. However, in the course of time normal bacteria redeveloped. These organisms were gram-negative and gave the biochemical reactions of *Salmonella typhosa*, but agglutination with typhoid antiserum gave only a very weak reaction. Hauduroy thought he was justified in diagnosing his culture as typhoid bacilli. He concluded that in polluted water filterable invisible forms of ordinary bacteria may occur.

Secondly, Hauduroy investigated the blood of a typhoid fever patient, drawn at the height of the infection. A portion of blood was transferred to broth; on the fifth day of incubation macroscopical granules were present, and a filtrate of this broth transferred to fresh broth again produced granules. The first broth tube containing the blood was turbid on the tenth day, and its turbidity was due to a growth of typhoid bacilli. This observation suggests according to Hauduroy that a filterable phase of the typhoid bacillus might have been present in the patient's blood.

If these few examples of positive filtration experiments, which easily could be multiplied, are analyzed, it will be seen that in each case the result is a different one. In Friedberger's experiments a mysterious virus-like agent was found which, however did not manifest itself in Gildemeister and Herzberg's investigations. Fejgin obtained similar results as Friedberger with typhoid. Hauduroy found granular and amorphous material, a result difficult to assess; yet it might have been an L culture. The retarded appearance of *S. typhosa* in his blood culture may be explained by the inhibiting effect of typhoid antibodies present in the patient's blood; whereas his diagnosis of the organism from spring water contains an element of uncertainty. It has therefore to be admitted that the results of the filtration experiments are not consistent. Part of these inconsistencies may be due to errors to which Frobisher (49) among others drew attention in an excellent paper. He carried out many elaborate control experiments concerning filtration. He showed that slow growing environmental bacteria may be responsible for some of the results of apparent positive filtration. The appearance of granules and filamentous or amorphous material, he pointed out, may be due to precipitations which frequently occur, if broth and especially serum broth, is incubated for prolonged periods. Furthermore, some gram-negative air contaminants may morphologically and biochemically resemble pathogens of the typhoid-coli group, and even positive agglutinations can be deceiving. Thus it cannot be denied that, quite apart from defective filters, filtration experiments involve "a variety of obscure sources of error".

Yet if filtration results are consistent and if the growth that develops from

the filtrate shows a characteristic morphological appearance, then it is justifiable to conclude that something which was in the original suspension, passed the filter. As Hadley, Delves and Klimeck's (59) studies meet these requirements, they will be described and discussed in detail. The authors worked with the Shiga bacillus and started their experiments by "setting into operation the dissociative reaction in the mother culture". This can be achieved by subjecting the culture to factors such as aging, metabolic products, temperature changes, variations of the physical state of the medium, various pH's, starvation, animal passage, or by adding substances to the medium such as salts, urea, dyes, antiseptics or by growing in normal or immune sera, in brief by all those adverse conditions which, in small doses, also stimulate L form production. Antibiotics, not yet discovered in 1931, would also have a similar influence. The authors subjected their *Shigella* culture first to one of the stimuli mentioned above, then filtered and spread several drops of the filtrate on a litmus lactose agar plate. If visible colonies developed during the first 48 hours, the plates were discarded, because it was concluded that ordinary bacterial forms had passed the filter. The 48-hr. plates, on which even microscopically no colonies were discovered, were washed off and the washings transferred to new plates. This was continued until six consecutive plates had been used. Eventually tiny and characteristic colonies appeared which could be discovered only under the microscope. These colonies were designated as G type colonies by Hadley. The letter G stands for gonidia. The diameters of these colonies varied between 0.004 and 0.2 mm. Morphologically G-colonies consist, according to Hadley, of granules of various sizes, coccal forms, large globules and swollen or deformed rods. Apparently the G type culture is grown with some difficulty and at first grows better on liquid than on solid media. If the cultures were transferred frequently, they usually reverted after a time into the Shiga bacillus.

For anyone who has had no previous experience with L cultures or with G cultures, the method by which Hadley obtained his G type cultures must seem obscure and liable to errors. However, when attempts have been made in my laboratory to isolate an L culture from *S. moniliformis* or from *S. necrophorus* on the special media devised for this purpose, three kinds of colonies have appeared on the plates: (a) large colonies which were mainly bacillary, (b) tiny L colonies which contained no bacillary forms, (c) intermediate colonies of all sizes from very tiny ones to the almost bacillary ones. These intermediate colonies contained many L forms, i.e. granules and bodies, but they also contained varying numbers of bacillary elements. They represent apparently the type of colony which Hadley designated as G type. Whereas the bacillary and particularly the L type colonies are stable, the G type colonies are variable. Hadley himself seems never to have cultured the L form, but he has postulated its existence, and we believe, it may have been on his plates, but was not demonstrable before the G type colonies developed. If we replace the expression "filterable virus form" with "L form", we understand that Hadley's deductions are in full agreement with our conception that the G colony is intermediate between the bacillary colony and the L colony. In Hadley's words: "The G type culture does

not comprise the filterable virus form (*L form*) of the Shiga bacillus, but it includes it, at least to a much greater degree than any other culture type of which we possess any knowledge. The filterable elements (*L elements*), at a certain stage of their development, do not manifest visible growth in or on any culture medium. In due time, however, and very gradually, they generate the visible granular and coccoid elements of the G type culture, which itself may be a growth of extreme delicacy. This type of culture, for a considerable time at least, may not only maintain itself, but also regenerate more of the filterable bodies (*L bodies*). Beyond a certain point in the development of the G culture, however, this ceases to be the case, and the culture begins its gradual ascent, through ontogeny, toward the normal type, and subsequently to the reproductively mature form. According to our view, the G type culture may thus be regarded as the intermediate culture state lying between the filterable virus stage (*L stage*) of the organism and the adult form." (Italicized words are mine)

It may be asked why Hadley could not demonstrate the L phase on his plates if it was present. Admittedly the L phase is visible and can be cultured, but it can only be demonstrated by very special fixing and staining methods or phase contrast microscopy, all of which have been developed since. Furthermore, if there is only a very delicate growth present, this may even today escape notice as the finest first growth of the L phase is very difficult to identify.

G type cultures have probably been observed much more frequently by bacteriologists than is realized. When material from patients is inoculated on plates and the incubated plates are examined microscopically, G type colonies are occasionally present. Impression preparations, made from these small colonies, reveal L elements such as granules and bodies of various sizes and shapes, but there are also bacillary elements present. These colonies are difficult to grow, and unless they revert into the bacillary stage they are often lost in the further passages. In contrast to these variable G colonies the stabilized L strains are extremely stable. In their filterability and morphology they resemble the organisms of the pleuropneumonia group. Already in 1931, Hadley had observed in regard to the bodies in the G cultures that "Some of them possess a striking resemblance to some of the bodies that have been pictured for the virus of contagious pleuropneumonia of cattle".

Hadley's results have never been fully confirmed (see his review (57), p. 154). This is not surprising, for it has been shown for *S. moniliformis* that the filterable granules, as a rule cannot be grown by themselves, unless the L culture has been adapted in long term passages to grow on artificial media. It is rather more remarkable that Hadley obtained positive results, and it would appear that he probably worked with particularly suitable strains and favorable conditions of culture.

In his review on "microbic dissociation" Hadley (55) expressed the opinion that a mechanism must exist which is responsible for the phenomenon of dissociation. When variation takes place on solid media, development of papillae frequently indicates that a new variety has grown up. The inhibiting factors that set in action the supposed mechanism must therefore be the same as those that

result in the formation of papillae. According to Hadley the unknown mechanism is connected with his postulated filterable phase. It is assumed here that this filterable phase is the L phase and that the mechanism is: passage through the L cycle with all that it involves. All known facts are in agreement with this assumption. For example, it has been shown by Barkulis (7) that *S. typhosa* is slightly inhibited in media containing any one of the following: L-rhamnose, L-arabinose, L-sorbose or L-fucose. The slightly inhibited colonies form papillae from which "mutants" can be isolated which are no longer inhibited. The mechanism of the inhibition was not studied by Barkulis, but Hadley (55) and Kritchewski and Ponomarewa (91) and myself have demonstrated that the papillae, formed by various organisms under the influence of carbohydrates, which they do not ferment in the first place, contain a great amount of highly pleomorphic cells, i.e., L forms. It may therefore be assumed that under these circumstances bacteria pass through an L cycle and produce by regeneration and possibly by mutation (128) a new variety able to ferment the primarily inhibiting substance. With a better chance of nutrition the new variety grows into secondary colonies, the papillae.

To sum up: The mechanism behind the phenomenon of dissociation is taken to be regeneration by means of the L cycle. For this reason all the stimuli that induce bacteria to dissociate, are also those that set the L cycle in action. The L phase produces a large amount of filterable granules and is therefore identical with Hadley's postulated filterable granular stage. Hadley's G colony is taken to be intermediate between an L colony and a bacillary colony.

(*b*) *Morphological observations.* Morphological investigations of bacteria and observations concerning a granular stage were carried out long before filtration experiments. Firtsch (44) studied "Variationserscheinungen" in "Vibrio Proteus". He described plasma globules ("Plasmakugeln") which he compared to the "Ferran bodies" of the cholera vibrio measuring 5 to 7μ in diameter. He emphasized that they were filled with granules. His most interesting remark is expressed as follows: "From the picture seen it would appear that these bodies arise from the fusion of filaments which form a loop or knot". This statement seems to express his direct observation, but under the influence of the monomorphic school of thought he continues: "Naturally, this behaviour cannot have anything to do with fructification". Firtsch is probably one of the first authors who have observed the fusion, by which L elements are produced, and his big globules must be interpreted as L bodies.

One of the best observers of the older generation of bacteriologists was Almquist (4, 5). He was the first to use a lowered temperature in order to stimulate bacteria to go into the regenerative cycle. His typhoid cultures, when kept at 10 C, produced very small, scarcely visible granules. When he filtered the cultures in this state he was sometimes able to obtain granular cultures which produced immune sera in rabbits. Almquist also observed the big forms which he described very appropriately as plasmodia. Furthermore, he found that branched forms are connected with this state.

The well known studies of Löhnis and Smith (98, 99) were carried out on

Azotobacter agile. They describe an amorphous stage of bacteria called "symplasm" in contrast to the "formed" ordinary bacteria. Their observation that the amorphous conglomeration reproduces bacteria seems correct, and it is justifiable to identify this amorphous stage with the L stage. Filterable forms are also included in Löhnis' rather complicated life cycle of *Azotobacter agile* which, although in agreement with some of the newer observations in the field of L forms, also seems to contain some fallacies.

Enderlein's book on "Bakteriencyclogenie" (39) presents a philosophical treatise rather than a discussion of scientific facts based on exact observations and is therefore not further discussed here.

Hort (72) studied organisms of the coli, typhoid, dysentery group by means of a microscope fitted with a warm stage. He observed the formation of very small granules which according to his illustrations seem to be similar to those which I (89) hold responsible for the initiation of the L growth by fusion. Mellon (101, 102, 103, 104) has been one of the main workers in the field of bacterial life cycles. He described the occurrence of big bodies (L forms) in various organisms and postulated the existence of a filterable granular phase. In his papers on microbic heredity he published very good photographs of bacteria apparently in the act of fusion and he interpreted them as a "primitive sexual process".

The life cycle theories of Löhnis, Enderlein, Hort and Mellon were criticised by Henrici (70) in a comprehensive and very good review. At the time he was quite correct in concluding: "We may sum up the present status of the problem by stating that bacteria in pure culture do show wide variations in the size and form and structure of their cells, but that the nature and significance of these variations are not known". Henrici included in his book an illustration which, unknown to him, presents extremely good pictorial evidence of the actual process of fusion in bacteria. He called it "Representative cells from a culture of the cholera vibrio at different stages of growth". Any unprejudiced biologist, however, must think of conjugation when he studies the illustration.

Extensive investigations on bacterial pleomorphism have been carried out by Kuhn and Sternberg (92). Kuhn studied his cultures on media containing lithium chloride. He interpreted the big "aberrent forms", which arose, as the cells of a parasite of the bacteria and designated them as "Pettenkoferia". He interpreted the minute granules which he also observed in his cultures as "spores" of the parasite. Preisz (122) followed in Kuhn's wake and published a fully and beautifully illustrated paper on the so-called bacterial parasite. His illustrations show L forms of all kinds as well as small granules formed by bacteria. The parasite theory is, of course, no longer tenable, as the L form is now becoming recognized as a phase of bacteria.

One phenomenon connected with the L phase should be mentioned here too. Branching forms have been observed in various species of bacteria which ordinarily grow in the rod shape. Gardner (50) described this phenomenon and called it "three point multiplication". As has been shown by me (89) and remarked upon by Hadley and Almquist, this process is closely connected with what is now called the L cycle. When L forms reproduce ordinary bacteria they

invariably produce amoeboid forms and divide by cutting off the often long pseudopodial endings. Thus, they simulate branched forms. Amoeboid forms, particularly when transferred to new media, transform into bacteria by division steps as Gardner has pointed out and illustrated.

It has been shown in the preceding chapter that if we regard the L cycle as a regeneration process, which also comprises a granular filterable form, it can be seen that the various authors who thought they were dealing with different phenomena were all describing various aspects of the same L cycle.

FILTERABLE FORMS AND BACTERIOPHAGE

Hauduroy (61, 62) reported results of experiments which at first seemed unusual, but which have since been frequently confirmed by him and other workers. He observed that a bacterial culture, lysed by bacteriophage and filtered through a candle, became slightly turbid after some time had elapsed, and eventually the Shiga bacillus reappeared in the filtrate. To explore this phenomenon further he incubated 20 tubes of filtrate for a considerable time. Two showed slight turbidity on the fifth day, two more became slightly turbid on the sixth day. Out of the remaining 16 a few developed turbidity much later, the rest remained clear. With a moderately active bacteriophage and the same culture of Shiga bacilli this experiment could be repeated many times and always with a similar result. In contrast to this from a Shiga broth culture, not subjected to the action of bacteriophage, a filtrate was obtained that remained sterile indefinitely. The bacteriophage filtrates which did not remain clear showed at first only opalescence, and the turbidity developed reluctantly. The opalescent and slightly turbid cultures were very different from an ordinary Shiga culture. They contained many small granules, isolated or in groups, filamentous material, and very few bacilli. When they developed further their growth in broth was either homogeneous or it consisted of agglutinated material or of a uniform sediment at the bottom of the tube. The secondary cultures differed, not only in growth habits, but also in biochemical reactions from the mother strain. They could be maintained by serial passage, yet grew often only in liquid and not on solid media. When they eventually reverted to the classical type after a number of passages they were usually bacteriophage resistant.

D'Herelle himself made a similar observation and together with Hauduroy (71) he contributed further interesting information. When the bacteriophage was weak, the secondary cultures appeared frequently and without much delay and the now resistant strains did not differ appreciably from the mother strain. When the bacteriophage was strong the secondary cultures were rare and developed only after considerable delay. The elements of which such a delayed culture consisted were mainly gram negative granules even when derived from staphylococci. Cultures of the granular type did not grow in ordinary broth but required albumen. They remained in the granular state without reverting. They sometimes persisted for long periods without transfers.

Kendall and Walker (80) studied how the spontaneous "come back" of bacteria in filtered bacteriophage solutions could be provoked. They assumed that

the bacteriophage caused a number of organisms to go into what they called the "invisible non-culturable" phase. This phase multiplied very little and fed on the proteins liberated from the lysed bacteria. The reappearance of ordinary bacteria in the bacteriophage solutions would be possible only if one postulates that filterable forms exist and (a) the bacteriophage is destroyed or loses its activity or (b) the newly growing bacteria are bacteriophage resistant. For even if the granular form passed the filter, if neither (a) nor (b) were fulfilled the bacteria could not develop further. In order to obtain secondary growth the authors employed methods supposed to curb bacteriophage activity. For example, exposure to methylene blue and sunlight, the addition of killed homologous bacteria, addition of specific anti-phage. Thus they found that the secondary bacteria had a better chance to develop in the filtrates. As they occasionally obtained secondary cultures from very highly diluted filtrates (1:10,000,000) they concluded that the number of filter passing forms can be very high.

In his review on the Twort-d'Herelle phenomenon, Hadley (56) discussed the filterable forms of bacteria which may be present in filtered bacteriophage suspensions. He emphasized that he was not referring to the occasional small filter passing ordinary bacteria, but he meant a specialized stage completely different from ordinary bacteria, i.e., the filterable phase. The key to Hadley's opinion on the relationship between bacteriophage and filterable forms of bacteria is found in the sentence: "It's (the bacteriophage's) most significant action is to transform cells, not to destroy them". In my opinion the bacteriophage acts primarily as a lytic factor and secondarily as a transforming agent. When bacteriophage:bacteria suspensions are spread on plates in such a way, that discrete plaques develop, it can easily be seen by the osmic acid-Giemsa, Bouin-Giemsa or phase contrast microscopy methods that a large amount of L forms develop at the very edges of the plaques, where the bacteriophage is in low concentration. The L phase is not lysed; it multiplies in the presence of bacteriophage. After some time the L bodies reproduce new young bacteria, some of which may have become resistant to bacteriophage and grow out into resistant secondary colonies. Therefore the bacteriophage can be classified with all those adverse factors that induce bacteria to go into the L phase and at the same time produce bacterial dissociation. It is quite feasible to suppose that the L granules pass filters and under favorable conditions reproduce the bacteria in the bacteriophage filtrates. Experiments to prove this are in progress.

CONCLUSIONS AND OUTLOOK

Many bacteria are able to assume a form in which they lack defined boundaries and constant size. This form of growth, called L phase, has a development of its own, which culminates in the production of large numbers of small granules which are on the borderline of ordinary microscopical visibility. It has been shown for the L phase of *S. moniliformis* that in a stabilized L strain these granular forms can be filtered through gradocol membranes and recovered from the filtrate in large numbers. The filtration analysis shows that the granular stage is of the same order of size as the larger viruses and the filterable organisms

of the pleuropneumonia group. So far filtration analysis has only been carried out in one species. The L phase has been demonstrated by growth and morphological methods in many bacterial organisms belonging to widely different groups. It seems to be a general feature of bacteria, which are able to occur in two phases, the bacterial and the L phase. Furthermore, it is possible that most bacteria, when existing in the L phase are filterable. It has been the task of this review to draw attention to the evidence contained in the literature which supports the existence of a filterable granular phase.

In pathogenic spirochetes a granular phase, probably endowed with special resistance to antibodies, and able to reproduce the spiral adult form, has been described by a number of authors. In acid-fast bacteria, particularly in the tubercle bacillus, the existence of a granular phase is widely assumed. Filtration experiments have so far not been consistently successful in the demonstration of such a phase. As we now know that the L phase is extremely difficult to grow from a single granule inoculum, it is not surprising that filtration experiments have so far failed. Yet it is hoped that filtration analysis will yield positive results a soon as the conditions of growth of the L phase have been further explored and media have been devised allowing a single granule inoculum to germinate. The morphological evidence so far available for the existence of a granular phase in acid-fast bacteria is more convincing than the filtration experiments. Though the whole cycle of the tubercle bacillus has not yet been completely revealed, the granular phase has been clearly shown by several authors. This phase may play an important role in various diseases of tuberculous origin.

In the literature dealing with various taxonomic groups the so-called "abnormal" forms such as big bodies, amorphous masses and granules have been widely described, though they have been interpreted diversely, but they are probably stages in the L cycle. It has also been pointed out that the appearance of these L forms is connected with the appearance of variations. This raises an important question: What is the significance of the L cycle? The L phase probably arises as the product of the fusion of small elements of bacterial origin. This fusion may be the primitive equivalent of a sexual union in higher organisms. Therefore the L cycle is regarded as a process of regeneration, probably involving gene recombination. It is stimulated in growing cultures whenever the balance of steady multiplication is upset, which can be caused by a change of environment, by a change of temperature and by many factors that produce slightly adverse conditions. Of course, in some bacteria such as *S. moniliformis* and *S. necrophorus* a stimulating cause is not known as they go through their L cycle whenever they grow. The L phase is usually only a transient stage of short duration; it reproduces the bacillary phase. As a regeneration has taken place the new bacteria may emerge as a different race showing morphological, antigenic or biochemical differences. If the special conditions of the environment are more favorable for the altered individuals, than they have been for the parent form, a new development of the altered kind will take place. Papillae (secondary colonies) may arise which may consist of organisms better adapted to the unfavorable conditions. One of the well known factors which produces new races is the bacteriophage. If

a suitable concentration of the bacteriophage is used, the lysis of the bacteria is followed up by the development of the L phase. The L phase seems to multiply unhindered by the action of weak bacteriophage and eventually reproduces a bacterial phase which may be phage-resistant and grow into resistant colonies.

To sum up: By a process of fusion and regeneration bacteria seem to be able to transform into a special phase, the L phase. This phase is difficult to demonstrate unless appropriate methods are used. It can reproduce by tiny granular forms which pass through filters that retain ordinary bacteria. This L phase is liable to arise under slightly unfavorable conditions. It may be more resistant to these conditions than the bacterial phase. When it reverts to the bacterial phase altered races may emerge, better adapted to the environment than the parent race.

However, our knowledge of the L phase is still fragmentary and many gaps will have to be filled. First of all media will have to be improved, so that cultures can be obtained from single granules. Only then the size of the granules and the number produced by the L forms of various bacteria can be determined. When cultures can be established with greater ease, the morphological stages of the L cycle can be studied by modern methods such as phase contrast microscopy, electron microscopy and the newer fixing and staining techniques. Studies of the resistance of the L phase and its pathogenic, serological and biochemical reactions will also be essential. Finally, therapeutic agents will have to be found which will clear the host of this most dangerous phase.

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